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Facultative polypeptide translocation allows a single mRNA to encode the secreted and cytosolic forms of plasminogen activators inhibitor 2

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Two forms of plasminogen activators inhibitor 2 (PAI-2) are synthesized by human and murine monocytes/macrophages: one accumulates in the cytosol, while the other is translocated into the endoplasmic reticulum, glycosylated and secreted. We show here that a single mRNA encodes both forms of PAI-2. Firstly, a single PAI-2 mRNA was detected by Northern blot hybridization and by RNase protection. Secondly, transfection of a PAI-2 cDNA led to the synthesis of both forms of PAI-2. Finally, *in vitro* translation of an mRNA transcript of the PAI-2 cDNA in the presence of microsomal membranes generated two topologically distinct forms of PAI-2. The cytosolic and secreted forms of PAI-2 do not result from the use of two translation start sites, since their synthesis initiates at the same AUG, in a sequence context that is conserved between the human and murine genes. Thus, the accumulation of one polypeptide into two topologically distinct cellular compartments can be achieved by facultative translocation.

Key words: monocytes/plasminogen activators/secretion/serpin/signal peptide

Introduction

The secretion of proteins requires their unidirectional translocation across a lipid bilayer from the site of mRNA translation, the cytosol, into the lumen of the rough endoplasmic reticulum (RER). The information responsible for this translocation resides in an N-terminal targeting element, the signal or leader sequence (Walter and Lingappa, 1986). Although signal sequences are highly degenerate, they are probably subjected to a strong selective pressure, since partial protein missorting is considered to be not only ineffectual but also, in the case of degradative enzymes, detrimental to cell survival. There are two known examples where the same protein functions both in the cytosol and outside the cell. The secreted and cytoplasmic forms of yeast invertase are encoded by a single gene and produced from two overlapping mRNAs; the larger mRNA codes for the secreted enzyme and its N-terminal signal sequence, while the shorter transcript initiates within the signal sequence and codes for the cytosolic invertase (Carlson *et al.*, 1983). The cytosolic and plasmatic forms of gelsolin are also generated by the alternative use of two promoters. The mRNA coding for the cytosolic form contains a unique 5′ untranslated region, derived from two exons, and its translation starts at the beginning of an internal exon, which is common to both mRNAs; the 5′ end of the mRNA coding for the plasma form derives from a unique exon which codes for an N-terminal signal sequence (Kwiatkowski *et al.*, 1988). After translocation is initiated, the signal peptides of most secreted proteins are cleaved in the lumen of the RER by a leader peptidase. Notable exceptions to this rule are two members of the superfamily of serine protease inhibitors (serpins; Carell and Travis, 1985), ovalbumin (Tabe *et al.*, 1984) and plasminogen activators inhibitor 2 (PAI-2) (Ye *et al.*, 1988), one of the specific inhibitors of plasminogen activators.

Plasminogen activators (PAs) are serine proteases that convert plasminogen, an inactive zymogen, into plasmin, a neutral protease of broad specificity (Danø *et al.*, 1985). The activity of PAs is controlled at multiple levels, including the regulated synthesis of PA-specific inhibitors (PAIs). One of these PAIs, originally purified from placenta (Kawano *et al.*, 1968) and now called PAI-2, was found to be synthesized by murine and human macrophages, by monocytic cell lines, and by several lines of different origin (Chapman *et al.*, 1982; Golder and Stephens, 1983; Genton *et al.*, 1987; Wohlwend *et al.*, 1987a,b). Interestingly, two forms of PAI-2 have been described: one form accumulates in the cell, and was recently found to be stored in the cytosol (A.Wohlwend and colleagues, in preparation), while the other, which is glycosylated, is secreted into the medium (Genton *et al.*, 1987; Wohlwend *et al.*, 1987a,b) without cleavage of a signal peptide (Ye *et al.*, 1988). The secreted (sPAI-2) and the cytosolic (cPAI-2) forms of PAI-2 have been shown to be functionally and immunologically indistinguishable (Genton *et al.*, 1987; Wohlwend *et al.*, 1987a), and enzymatic removal of the polysaccharide portion of sPAI-2 was found to yield a protein that co-migrated with cPAI-2 (Ye *et al.*, 1988). The bi-topological distribution of PAI-2 appears to be a unique property of this protein and does not reflect a general deficiency in the protein translocation machinery of certain cell lines, since other secreted proteins such as lysozyme are efficiently translocated by the same cells which produce the two forms of PAI-2 (Gupta *et al.*, 1985). cDNA and genomic clones of human PAI-2 have been isolated by several groups (Schleuning *et al.*, 1987; Webb *et al.*, 1987; Ye *et al.*, 1987, 1989); the published PAI-2 sequences differ by a small number of base changes that probably reflect the presence of two alleles, although the existence of two related PAI-2 genes was not excluded (Ye *et al.*, 1989).

In this communication, we have investigated the mechanism responsible for the synthesis of the cytosolic and secreted forms of PAI-2. Our results show that there is only one PAI-2 mRNA, and that this mRNA encodes both forms of the protein. The facultative translocation of PAI-2 represents a mechanism to generate topologically distinct

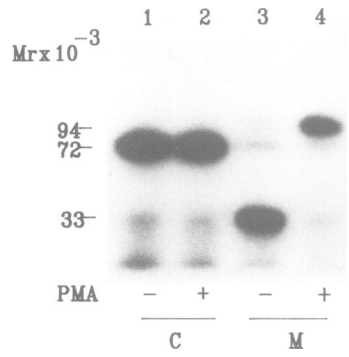

Fig. 1. Production of cPAI-2 and sPAI-2 by U937 cells. Cells were cultured for 20 h in control medium (**lanes 1** and **3**) or in medium supplemented with 30 ng/ml PMA (**lanes 2** and **4**). Samples (10 μl) of cell extracts (C, **lanes 1** and **2**) and conditioned media (M, **lanes 3** and **4**) were incubated for 1 h at 4°C with 2 ng of M_r 33 000 ^{125}I-labeled uPA (5 μl) and analyzed by SDS−PAGE and autoradiography. Unreacted uPA migrates as a single M_r 33 000 band (not shown). The M_r of uPA−PAI-2 complexes were calculated from the position of markers electrophoresed in a parallel lane and stained with Coomassie blue.

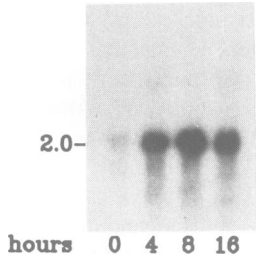

Fig. 2. Northern blot analysis of PAI-2 mRNA in PMA-treated U937 cells. U937 cells were incubated for the indicated times in medium supplemented with 50 ng/ml PMA. Total cellular RNA was isolated and electrophoresed in a 1% agarose gel (4 μg/lane). Filters were hybridized with a PAI-2 cRNA probe and exposed for 24 h at 20°C. The size of the PAI-2 mRNA was calculated from the position of rRNAs electrophoresed in a parallel lane and stained with ethidium bromide.

forms of a single polypeptide, which can exert their activity in different physiological situations.

Results

A single PAI-2 mRNA is present in U937 cells

Cells from the human monocytic U937 line synthesize and contain large amounts of a cell-associated, non-glycosylated form of PAI-2 (cPAI-2; Genton *et al.*, 1987; Wohlwend *et al.*, 1987a; Ye *et al.*, 1988). This protein reacts with M_r 33 000 ^{125}I-labeled urokinase-type PA (uPA) to form a covalent complex of M_r 72 000 (Figure 1, lane 1). We have recently shown that cPAI-2 is a soluble protein which is stored in the cytosol (A.Wohlwend and colleagues, in preparation). Only small amounts of cPAI-2 are detected in the culture medium (lane 3), and this could result from the lysis of a small fraction of the cells during culture or cell manipulation. The differentiation of U937 cells induced by the tumor promoter PMA is accompanied by an increase in total PAI-2 production, and a significant amount of the newly synthesized PAI-2 is secreted in a glycosylated form (sPAI-2; Genton *et al.*, 1987; Wohlwend *et al.*, 1987a; Ye *et al.*, 1988). The complex formed between sPAI-2 and ^{125}I-labeled uPA migrates with an M_r of 94 000 (lane 4) and is clearly resolved from that obtained with cPAI-2.

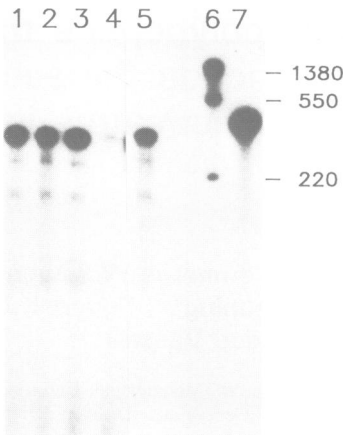

Fig. 3. RNase protection analysis of PAI-2 mRNA in U937 cells. Total RNA was isolated from control cultures (**lane 1**), and from cultures incubated with PMA for 4 h (**lane 2**) and 8 h (**lane 3**). To analyze equivalent amounts of PAI-2 mRNA, samples from control and PMA-treated cells contained 5 and 0.5 μg of cellular RNA respectively. **Lane 4**, no cellular RNA; **lane 5**, 100 pg of PAI-2 mRNA transcribed *in vitro* from pDB5702. Total RNA was kept constant (5 μg/sample) by the addition of tRNA. After hybridization to a purified ^{32}P-labeled PAI-2 cRNA probe (from pDB4708) and digestion with pancreatic RNase, the hybrids were denatured and electrophoresed in a 9% polyacrylamide gel. **Lane 6**, run-off transcript markers; **lane 7**, unhybridized probe (23 nt are encoded by the vector and not complementary to PAI-2 mRNA).

To determine whether the synthesis and secretion of sPAI-2 results from the induction of a different PAI-2 mRNA, total RNA was extracted from U937 cells incubated with PMA for various lengths of time and from control cultures. The RNAs were first analyzed by Northern blot hybridization (Figure 2). As expected (Schleuning *et al.*, 1987; Webb *et al.*, 1987; Ye *et al.*, 1987), the increased production of PAI-2 in PMA-treated cells is accounted for by an increased mRNA content, which is detectable at 4 h and persists for at least 16 h. At this level of resolution, only a single PAI-2 mRNA could be detected at all times.

To refine the analysis of PAI-2 mRNA in U937 cells, the cellular RNAs described above were subjected to RNase protection experiments. A human PAI-2 cDNA has been isolated from a U937 cell library (Schleuning *et al.*, 1987) and we first used as a probe a 322 nt cRNA which covers 21 nt of the 5′ untranslated region and the first 301 nt of the coding region. After digestion of the non-hybridized RNAs with pancreatic ribonuclease (Figure 3), or with ribonuclease T1 (not shown), the protected cRNAs were electrophoresed and revealed by autoradiography. A single major protected cRNA is detected after hybridization to RNAs from both control U937 cells (lane 1) and PMA-treated cultures (lanes 2 and 3); the size of this protected cRNA fragment corresponds to the length of PAI-2 sequences in the probe (lane 7). Similar results were obtained with two other cRNA probes, which cover the rest of the PAI-2 mRNA coding region (not shown).

A few minor cRNA fragments were often apparent in these protection experiments. These minor species are also observed when a purified synthetic PAI-2 mRNA, resulting from *in vitro* transcription of the cDNA insert, is hybridized to the probe (lane 5). Thus, the minor fragments are probably

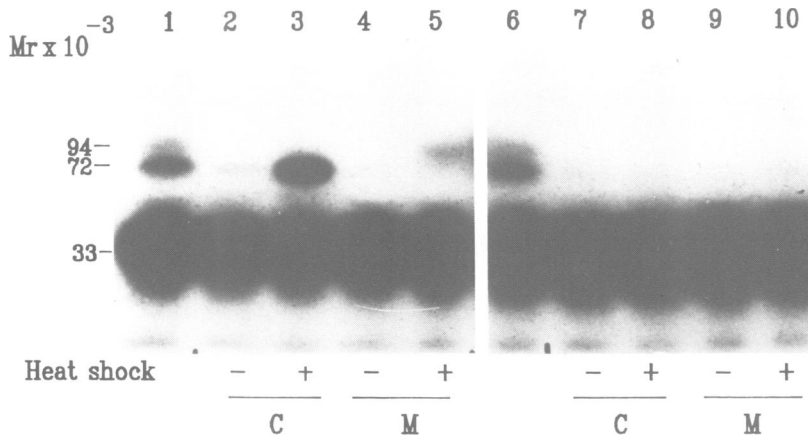

Fig. 4. Production of cPAI-2 and sPAI-2 in cells transfected with an inducible PAI-2 cDNA. Cells from a *neo*^{R} clone (**lanes 2–5**) and from the parent cell line (**lanes 7–10**) were maintained at 37°C for 22 h (**lanes 2, 4, 7** and **9**) or subjected to a 2 h heat-shock at 43°C and cultured for 20 h at 37°C (**lanes 3, 5, 8** and **10**). Samples of cell extracts (**lanes 2, 3, 7** and **8**) and conditioned media (**lanes 4, 5, 9** and **10**) were incubated with M_r 33 000 ^{125}I-labeled uPA and analyzed by SDS–PAGE and autoradiography. **Lanes 1** and **6**, a mixture of sPAI-2 and cPAI-2 purified from U937 cells (Wohlwend *et al.*, 1987a) were incubated with M_r 33 000 ^{125}I-labeled uPA to provide size markers.

artefacts of the ribonuclease digestion and are unlikely to reflect the presence of other PAI-2 mRNA species in U937 cells.

These results suggest that a single PAI-2 mRNA may direct the synthesis of the cytosolic and secreted forms of PAI-2.

A transfected PAI-2 cDNA encodes both forms of the protein

To demonstrate directly that the cloned PAI-2 cDNA contains all the information required to direct the synthesis of both cPAI-2 and sPAI-2, the cDNA insert was linked to an inducible heat-shock promoter, in a plasmid that also carries a *neo*^{R} gene (Dreano *et al.*, 1987). This construct was transfected in WISH cells, a human amnion cell line which does not produce detectable levels of PAIs (Figure 4, lanes 7–10) and PAs (not shown). Several G418^R clones were isolated and 12–18 of these clones were found to produce PAI-2 after heat-shock. This is illustrated for one of the clones in Figure 4. After induction of the transfected gene, PAI-2 is recovered in the cell lysate (lane 3); this inhibitor forms an M_r 72 000 covalent complex with ^{125}I-labeled uPA which co-migrates with that formed by cPAI-2 purified from U937 cells. PAI-2 is also secreted in the culture medium (lane 5; see also Figure 6A, lane 1), and this secreted PAI-2 forms an M_r 94 000 covalent complex with ^{125}I-labeled uPA similar to that formed by sPAI-2 purified from the culture medium of PMA-treated U937 cells. In uninduced cultures, the cells contain lower amounts of cPAI-2 (lane 2), and very little PAI-2 is detected in the culture media (lane 4). No PAI is detected in cultures of non-transfected parent WISH cells (lanes 7–10). The identity of the inhibitors present in the cell lysate and culture medium of the transfected cells was further verified by immunoprecipitation with an anti-PAI-2 antiserum which recognizes both sPAI-2 and cPAI-2 (Figure 5). Attempts to change the ratio of sPAI-2 and cPAI-2 in the transfected cells were unsuccessful; in particular, stimulation of heat-shocked cultures with PMA, which leads to an increased recovery of sPAI-2 in the culture medium of U937 cells (Figure 1), and of mouse peritoneal macrophages (Wohlwend *et al.*, 1987b), had only a marginal effect on total PAI-2 production,

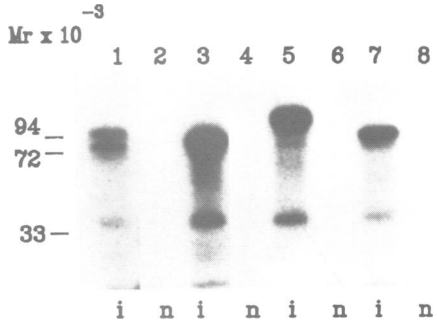

Fig. 5. Immunoprecipitation of PAI-2 in transfected cells. Cells from the clone described in Figure 4 were induced for 2 h at 43°C and cultured for 20 h at 37°C. The conditioned medium (**lanes 1** and **2**) and the cell extract (**lanes 3** and **4**) were concentrated 10-fold by lyophilization and desalted by chromatography on G-50 Sephadex in PBS. Appropriate dilutions of PMA-treated U937 cells conditioned medium (**lanes 5** and **6**) and cell extract (**lanes 7** and **8**) were processed in parallel. After incubation with M_r 33 000 ^{125}I-labeled uPA, the samples were immunoprecipitated with an anti-PAI-2 antiserum (Kruithof *et al.*, 1986; 1/30 final dilution) or with a non-immune rabbit serum. The immune complexes were bound to *Staphylococcus aureus*, washed and eluted as described (Wohlwend *et al.*, 1987a). **Lanes 1, 3, 5** and **7**, anti-PAI-2 antiserum (i); **lanes 2, 4, 6** and **8**, non-immune serum (n). The small amount of free M_r 33 000 ^{125}I-labeled uPA in lanes 1, 3, 5 and 7 results from hydrolysis of the uPA–PAI-2 complexes during sample preparation.

and did not increase the secretion of sPAI-2 (not shown).

cPAI-2 has been localized to the cytosol of U937 cells and of freshly isolated human monocytes (A.Wohlwend and colleagues, in preparation). To show that cPAI-2 synthesized in transfected WISH cells also accumulates in the cytosol, heat-induced cells were homogenized and subjected to differential centrifugation. An aliquot of each fraction, corresponding to 10^4 cells, was treated with the detergent Triton X-100, to release membrane-enclosed proteins, and assayed for the presence of PAI-2 by incubation with ^{125}I-labeled uPA (Figure 6, panel A). Essentially all the PAI-2 present in the postnuclear supernatant (lane 2) is recovered in the cytosol (lane 6), and there are only traces of PAI-2 in the membrane-enclosed compartments (lanes 3–5). This cytosolic cPAI-2 was sensitive to protease

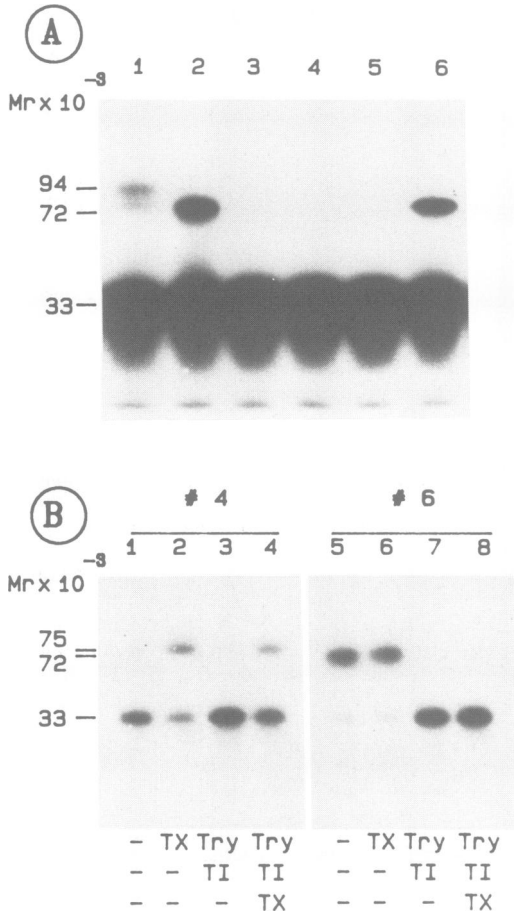

Fig. 6. Cell fractionation of PAI-2 in transfected cells. Cells from the clone described in Figure 4 were induced for 2 h at 43°C and cultured for 20 h at 37°C. (**Panel A**) The conditioned medium (**lane 1**) was collected and the cells fractionated as described (A.Wohlwend and colleagues, in preparation). Briefly, the cells (2.5×10^7 cells) were detached with EDTA and homogenized in 0.25 M sucrose; the post-nuclear supernatant (**lane 2**) was fractionated into: the 10 000 *g* pellet (**lane 3**), the 43 000 *g* pellet (**lane 4**), the 100 000 *g* pellet (**lane 5**) and the 100 000 *g* supernatant (**lane 6**). Aliquots of each fraction, corresponding to 10^4 cells, were incubated with M_r 33 000 ^{125}I-labeled uPA in the presence of 0.5% Triton X-100 and analyzed by SDS–PAGE and autoradiography. (**Panel B**) The 43 000 *g* (**#4, lanes 1–4**; 10^5 cells equivalent/lane) and 100 000 *g* supernatant (**#6, lanes 5–8**; 10^4 cells equivalent/lane) were subjected to protease–detergent treatment. **Lanes 1** and **5**, no addition. **lanes 2** and **6**, 0.2% Triton X-100; **lanes 3** and **7**, trypsin (Try) digestion (17 μg/ml for 60 min at 37°C), followed by trypsin inactivation with 50 μg/ml soybean trypsin inhibitor (TI); **lanes 4** and **8**, same as lanes 3 and 7, followed by the addition of Triton X-100 (0.2%). All samples were finally incubated with M_r 33 000 ^{125}I-labeled uPA and analyzed by SDS–PAGE and autoradiography.

digestion in the absence of detergent (Figure 6B, lane 7), and the addition of detergent to the protease-treated cytosol did not release any detectable PAI-2 (lane 8). Thus, the cytosolic cPAI-2 is not stored in a membrane-enclosed compartment in the transfected cells. In the Golgi-enriched fraction (Figure 6A, lane 4), small amounts of a membrane-enclosed form of PAI-2 (mPAI-2) were also detected (Figure 6B, lanes 1–4). This mPAI-2, which has a slightly slower mobility than cPAI-2 and is probably a glycosylation intermediate in sPAI-2 synthesis, constitutes 3–5% of the total PAI-2 in cell lysates. Most of mPAI-2 can only react with ^{125}I-labeled uPA to form a covalent complex after addition of detergent (cf. lanes 1 and 2), and is protected from protease digestion in the absence of detergent (lane 4).

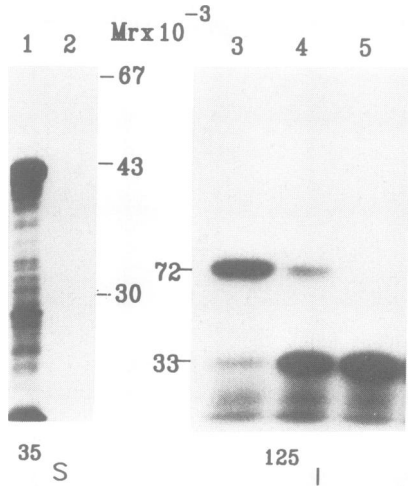

Fig. 7. *In vitro* synthesis of a functional PAI-2 indistinguishable from cPAI-2. Capped PAI-2 mRNA transcribed *in vitro* from pDB5702 was translated in a wheat germ extract in the presence of [^{35}S]methionine (**lane 1**); **lane 2**, control translation in the absence of exogenous template. Part of the samples were analyzed directly by SDS–PAGE and fluorography (**lanes 1** and **2**). Each reaction mixture was diluted and incubated with M_r 33 000 ^{125}I-labeled uPA. **Lane 3**, parallel incubation with a U937 cell extract as a source of cPAI-2; **lane 4**, *in vitro* synthesized PAI-2; **lane 5**, control translation. The samples were analyzed by SDS–PAGE under reducing conditions as described in Materials and methods, and ^{125}I-labeled uPA and uPA–PAI-2 complexes were detected by autoradiography of the wet gel.

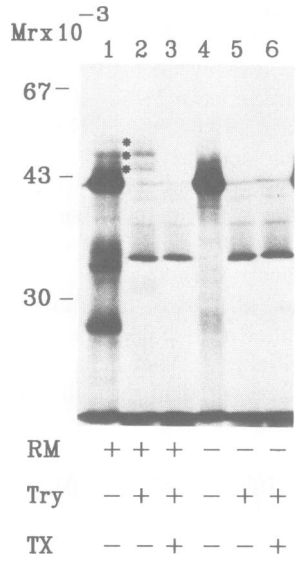

Fig. 8. *In vitro* translocation of PAI-2 in the presence of microsomal membranes. *In vitro* synthesized PAI-2 mRNA was translated in a wheat germ extract in the presence (**lanes 1–3**) or in the absence (**lanes 4–6**) of canine pancreas microsomal membranes (RM); the M_r 28 000 and 32 000 bands in lane 1 are probably due to the presence of contaminating proteases in the RM. After 90 min at 26°C, translation was terminated by the addition of 100 μg/ml pancreatic RNase. Aliquots were digested for 30 min at 0°C with 1 mg/ml trypsin (Try) in the absence (**lanes 2** and **5**) or in the presence (**lanes 3** and **6**) of 0.4% Triton X-100 (TX); the M_r 33 000 band in lanes 2, 3, 5 and 6 is a trypsin degradation intermediate of PAI-2. The samples were analyzed by SDS–PAGE and fluorography. The position of marker proteins electrophoresed in a parallel lane is indicated.

These results rule out the possibility that sPAI-2 and cPAI-2 are the products of two related genes. In addition, they indicate that a single mRNA species, in the form of a cDNA insert, can direct the synthesis of both the cytosolic and the secreted forms of PAI-2 *in vivo*.

Fig. 9. Sequence comparison in the N-terminal region of human and murine PAI-2. The nucleotide and deduced amino acid sequence of a human PAI-2 cDNA is from Schleuning *et al.* (1987). The isolation and sequencing of a murine PAI-2 cDNA is described in Materials and methods. Identical amino acids are marked by vertical lines.

In vitro synthesis and translocation of PAI-2

The results discussed above do not entirely exclude the possibility that a form of alternative splicing, exon removal (Breitbart *et al.*, 1987), could generate low levels of a shorter mRNA which would encode one of the two forms of PAI-2. Therefore it seemed important to determine whether a synthetic transcript of the PAI-2 cDNA could direct the synthesis of both cPAI-2 and sPAI-2 *in vitro*. Translation of this purified PAI-2 mRNA in a wheat germ lysate yields a major M_r 42 000 product (Figure 7, lane 1); PAI-2 synthesized *in vitro* is active since it reacts with ^{125}I-labeled uPA to form a covalent complex (lane 4) which co-migrates with that formed by cPAI-2 present in U937 cell extracts (lane 3). When the translation is performed in the presence of microsomal membranes, three additional bands (*) with higher apparent M_rs are detected (Figure 8, cf. lanes 1 and 4), a result compatible with the presence of three N-glycosylation sites in PAI-2 (Ye *et al.*, 1988). None of these bands are synthesized in the absence of PAI-2 mRNA (not shown). Trypsin digestion of the translation products shows that these additional bands are contained in a membrane-enclosed compartment, since they are only susceptible to proteolysis after addition of detergent (cf. lanes 2 and 3). The *in vitro* translocation of PAI-2 in this system was not very efficient, and a comparable low efficiency was also obtained with a purified murine PAI-2 mRNA (not shown). Although this observation is compatible with the notion that the sequence element which directs PAI-2 translocation *in vivo* may be inefficient, the interpretation of *in vitro* translocation efficiencies is difficult and can be misleading. Indeed, control experiments showed that murine uPA, which is efficiently translocated *in vivo* (Belin *et al.*, 1984), was also translocated very efficiently *in vitro*; however, the fusion protein of Sendai virus, which is efficiently translocated *in vivo* and with some microsomal membrane preparations *in vitro* (Vidal *et al.*, 1989), was translocated with the same low efficiency as PAI-2 in our *in vitro* translocation system (not shown). Finally, PAI-2 synthesized *in vitro* in the absence of membranes was subjected to N-terminal amino acid sequencing. The detection of a [^{35}S]methionine at position 1, and of [^{3}H]leucine at positions 4 and 10 (not shown) confirm that the synthesis of cPAI-2 initiates at the first AUG in the PAI-2 mRNA (Figure 9). The same results have been obtained with sPAI-2, which is secreted without cleavage of a signal peptide (Ye *et al.*, 1988).

These results indicate that translation initiation at the first AUG of PAI-2 mRNA leads to the synthesis of the two topologically distinct forms of the protein, cPAI-2 and sPAI-2. Thus, the sequence element that directs PAI-2 translocation must function in a facultative way.

The N-terminal sequence of PAI-2 is highly conserved

The N-terminal sequence of human PAI-2 is hydrophobic (Figure 9) and could therefore mediate sPAI-2 translocation; however, a comparison with other eukaryotic signal sequences reveals two unusual features: the N-terminal polar region contains two negatively charged residues (E_2 and D_3) and the hydrophobic region is interrupted twice by asparagine residues (N_8 and N_{14}). Murine macrophages, like their human counterparts, also synthesize two forms of PAI-2: a cytoplasmic, non-glycosylated, cPAI-2, and a secreted, glycosylated, sPAI-2 (Wohlwend *et al.*, 1987b). In an attempt to evaluate the possible relevance of the unusual N-terminal sequence of human PAI-2, we have isolated and sequenced a murine PAI-2 cDNA. The human and murine N-terminal sequences are compared in Figure 9. Although a glutamate is replaced by an aspartate (position 3), the two negative charges at positions 2 and 3 are conserved, as are the two asparagine residues (positions 8 and 14). Thus, while these two genes show a number of differences in their nucleotide and amino acid sequences, most of the amino acid changes are conservative, and the features that distinguish the N-terminal sequence of PAI-2 from most signal sequences have been conserved.

Discussion

In this communication, we have explored the mechanism that generates two topologically distinct forms of the serpin PAI-2. The secreted, glycosylated sPAI-2 and the cytosolic cPAI-2 are the products of a single gene. Likewise, the cytosolic and secreted forms of yeast invertase (Carlson *et al.*, 1983), and of mammalian gelsolin (Kwiatkowski *et al.*, 1988), are also the products of single genes. However, the strategy adopted in these three situations is strikingly different. The invertase and gelsolin genes are transcribed into two overlapping mRNAs, one encoding the cytosolic protein and the other the secreted one, while there is only one PAI-2 mRNA that encodes both forms of PAI-2. Thus, differential protein targeting may also occur by a co- or post-translational mechanism.

Initiation at two in-phase start codons can generate proteins differing only at their N-terminus and which could be targeted to different cellular compartments. However, the possibility that the PAI-2 mRNA is bicistronic (Strubin *et al.*, 1986; Prats *et al.*, 1989) can be excluded for a number of reasons. The 5′-proximal AUG is in a favorable sequence context (A**AUG**G), and is therefore expected to be the only functional initiation codon, according to the scanning model of eukaryotic translation initiation (Kozak, 1989). The PAI-2 sequence is identical between positions −11 and +5

in the human and mouse genes; in contrast, the other internal AUG codons are not conserved and their context appears less favorable. Also, truncated proteins initiated at internal AUGs would lack structural elements that are conserved in all serpins (Carell and Travis, 1985; Ye *et al.*, 1987). Finally, radiochemical sequencing of sPAI-2 (Ye *et al.*, 1988) and of *in vitro* synthesized PAI-2 identified methionine and leucine residues at the positions predicted by translation initiation at the first AUG of the human PAI-2 mRNA.

Several mechanisms could account for the partition of PAI-2 between the cytosolic and secretory compartments. (i) The N-terminal region of PAI-2 may constitute an unfavorable signal sequence which interacts only loosely with the signal recognition particle and/or the translocation machinery. Such a loose interaction would be compatible with a model which suggests that newly synthesized proteins partition kinetically between export-competent and export-incompetent conformations, and implicitly predicts the existence of proteins partitioning like PAI-2 (Gierasch, 1989; Randall and Hardy, 1989). (ii) All the newly synthesized PAI-2 may interact with the RER and initiate translocation; at a later stage, translocation of the molecules destined to the cytosol would be aborted. Such an abortive translocation has been demonstrated recently *in vitro* for the hepatitis B pre-core protein (Garcia *et al.*, 1988). The distinction between the first two mechanisms appears difficult since the putative signal sequence of PAI-2, unlike that of the pre-core protein, is not cleaved by the leader peptidase. It is thus not possible to discriminate between PAI-2 molecules that have not initiated translocation, and those which would result from an abortive translocation event. (iii) A third mechanism would require the post-translational translocation of a fraction of the newly synthesized PAI-2. It has been shown that heat-shock-like proteins can facilitate protein unfolding (Pelham, 1986) and post-translational translocation across yeast microsomes (Chirico *et al.*, 1988; Deshaies *et al.*, 1988). This mechanism may account for the release of a fraction of cPAI-2 in the extracellular space. Such post-translational translocations may also allow the release of IL-1 (Young *et al.*, 1988) and bFGF (Prats *et al.*, 1989), two cytokines that have no known signal sequences and do not appear to follow the classical secretory pathway. However, the results of pulse-chase experiments argue against such a mechanism for the generation of sPAI-2: the majority of newly synthesized cPAI-2 remains associated with the cells for several hours, while the intracellular glycosylation precursors of sPAI-2, which can be detected *in vivo* after a short 15 min labeling, are rapidly processed and secreted out of the cells (Ye *et al.*, 1988).

A number of structural elements may be responsible for the facultative translocation of PAI-2. By analogy with ovalbumin, which is also secreted without cleavage of its signal sequence, it has been postulated that the second hydrophobic region of PAI-2 (positions 25–46) accounts for its translocation (Ye *et al.*, 1988). However, ovalbumin is not known to accumulate in the cytosol of hen oviducts, and what appears salient about PAI-2 is the efficient synthesis of a functionally active cytosolic form. The N-terminal region of PAI-2 (positions 1–22) harbors some of the characteristics of known signal sequences (von Heijne, 1986), and was proposed to mediate the secretion of sPAI-2 (Schleuning *et al.*, 1987). The presence of two negatively charged residues near the N-terminus and of two asparagines within the hydrophobic core was unexpected, and could well account for the facultative translocation of PAI-2. Indeed, a computer search of published signal sequences detected only one set of sequences with two N-terminal negatively charged residues, those of the immunoglobulin K light chains of the V_k21 group (Burstein and Schechter, 1978; Heinrich *et al.*, 1984), and only a few sequences with asparagine in their hydrophobic core, including that of bovine prolactin (Sasavage *et al.*, 1982); there is no known example of a functional signal sequence containing two asparagine residues in the hydrophobic core. Since immunoglobulin chains and prolactin are not known to accumulate in the cytosol, their signal sequences must allow efficient translocation into the RER. An evaluation of the possible contribution of the two N-terminal negatively charged residues and of the two asparagines to the facultative translocation of PAI-2 *in vivo* must await the systematic analysis of single and multiple site-directed mutants.

It is generally assumed that translocation of proteins into the lumen of the RER is a constitutive property which is not subjected to modulation by cellular effectors. However, the distribution of PAI-2 in U937 cells appears to be influenced by the PMA-induced differentiation of these cells. In unstimulated cells, most of the newly synthesized PAI-2 accumulates in the cytosol, while after PMA treatment, PAI-2 distributes approximately evenly between the cytosolic and secretory compartments (Genton *et al.*, 1987; Wohlwend *et al.*, 1987a; Ye *et al.*, 1988). Although the half-life of secreted sPAI-2 has not been determined, these observations suggest the possibility that PMA increases not only the synthesis of both forms of PAI-2, but also the efficiency of secretion of sPAI-2. In addition, the relative distribution of PAI-2 in the two compartments may also depend on the cell type. In the transfected WISH cells, as well as in HT1080 fibrosarcoma cells (Medcalf *et al.*, 1988), only a small fraction of the total PAI-2 is secreted into the medium, while in PMA-treated U937 cells the amounts of newly synthesized cPAI-2 and sPAI-2 are approximately equivalent.

The accumulation of cPAI-2 in the cytosol of monocytes and macrophages presents an apparent paradox: its only known targets, uPA and tissue-type PA, are extracellular proteases, and cytosolic enzymes of similar specificity have not been identified. This suggests that cPAI-2 and sPAI-2 may exert their activity in the extracellular milieu, albeit at different stages of the inflammatory response. During the initial phase of an inflammatory reaction, polymorphonuclear phagocytes release a number of products, including cytotoxic oxygen radicals. The release of cPAI-2 from neighboring tissue macrophages would limit PA-catalyzed plasminogen activation and allow sufficient fibrin deposition for the healing of the inflammatory lesion to initiate. A similar mechanism has been proposed to account for the release of IL-1 by monocytes and macrophages (Young *et al.*, 1988). While the secretion of sPAI-2 by macrophages, which is induced by cytokines and other cellular effectors, requires on-going RNA and protein synthesis (Wohlwend *et al.*, 1987a,b), the large amounts of cPAI-2 stored in the cytosol of these cells may represent a reservoir of anti-fibrinolytic activity that can be mobilized under conditions of cell suffering where PAI-2 gene activation and biosynthesis would be restricted.

Secreted proteins have not usually been detected in the cytosol, and the finding of cytosolic proteins outside the cell

is pervasively used as an index of cell death. It is now known that a single polypeptide can accumulate both in the cytosol and outside the cell. Multiple transcription initiation sites and/or splicing patterns provide one mechanism to achieve the targeting of one protein into topologically distinct compartments. The facultative translocation of PAI-2 described here illustrates another mechanism to achieve this topological diversity.

Materials and methods

Cell culture

Culture of U937 cells, PMA-induced differentiation in serum-free medium supplemented with 1 mg/ml BSA and the collection of cell lysates and conditioned media were performed as described (Wohlwend *et al.*, 1987a). Cells of the WISH human cell line (ATCC-CCL25) were cultured and transfected as described (Dreano *et al.*, 1987). Heat treatments were performed with subconfluent cultures in serum-free medium; after 2 h at 43°C, fresh medium supplemented with 1 mg/ml BSA was added to the cells. Media and cell extracts were collected as above.

Analysis of uPA–PAI-2 complexes

Labeling of purified uPA, complex formation, immunoprecipitation and elution of immune complexes, SDS–PAGE and autoradiography have been described (Wohlwend *et al.*, 1987a). For the functional analysis of *in vitro* synthesized PAI-2, 3 μl of translation mixture was diluted with 7 μl of phosphate-buffered saline prior to the addition of 2 ng of ^{125}I-labeled uPA (total vol. 15 μl). Mixing experiments with sPAI-2 or cPAI-2 from U937 cultures have shown that 1:10 and 1:3 dilutions of *in vitro* translation mixtures in the absence of exogenous mRNA do not affect the extent of complex formation with uPA. After complex formation, 15 μl of 2-fold concentrated sample buffer supplemented with 5% β-mercaptoethanol were added and the samples were incubated for 15 min at 65°C prior to SDS–PAGE. This procedure prevented the occasional splitting of uPA–PAI-2 complexes into a closely spaced doublet (Kruithof *et al.*, 1986; Wohlwend *et al.*, 1987a,b).

RNA isolation and analyses

Isolation of cellular RNA and Northern blot hybridizations were performed as described (Busso *et al.*, 1986). For the RNA protection assays (Melton *et al.*, 1984), the indicated amounts of cellular RNA and tRNA were lyophilized and resuspended in 30 μl of hybridization mixture containing 5–10 ng of purified cRNA (1–2 × 10^5 c.p.m.). After 12–14 h at 40°C, the samples were digested for 1 h at 25°C with pancreatic RNase (0.5 μg/μg of total RNA) and processed as described (Belin *et al.*, 1987).

Plasmid construction and in vitro transcription with SP6 RNA polymerase

pDB4707 contains the 5′-proximal 794 bp *Eco*RI–*Xba*I fragment of human PAI-2 cDNA clone J.7 (Schleuning *et al.*, 1987) subcloned in pSP64 (Melton *et al.*, 1984); this plasmid was linearized with *Eco*RI and transcribed with SP6 RNA polymerase to generate the cRNA probe used in Northern blot hybridizations. pDB4708 was obtained by digestion of pDB4707 with *Pst*I and self-ligation; this plasmid contains only the 5′-proximal 322 nt of the human PAI-2 cDNA. pDB5702 contains the 1880 bp cDNA insert of human PAI-2 cDNA clone J.7 subcloned in the sense orientation into the *Eco*RI site of pSP65. pHS-PAI-2 was constructed by ligating the full-length PAI-2 cDNA insert of J.7 (filled-in with the Klenow fragment of DNA polymerase) to the vector portion of plasmid p17hGHneo (Dreano *et al.*, 1987) digested with *Bam*HI and filled-in with the Klenow fragment of DNA polymerase; recombinant plasmids were identified by hybridization to the PAI-2 cRNA probe from pDB4707, and the orientation of the insert determined by restriction enzyme analysis.

For RNA protection assays, cRNAs were labeled with 40 μCi of [^{32}P]UTP (100 pmol) in the presence of 1 nmol of unlabeled UTP; full-length transcripts were purified from 5% polyacrylamide, 7 M urea gels by electroelution in dialysis bags, phenol–chloroform extractions and ethanol precipitation. For *in vitro* translation, mRNAs were synthesized in the presence of 500 μM cap analog (Pharmacia), 50 μM GTP, 500 μM of ATP, CTP and UTP, and 3–10 μCi of [^{32}P]UTP.

In vitro translation

Wheat germ extracts were prepared as described (Dobberstein and Blobel, 1977) and used at 35% final concentration. Canine pancreas microsomes and [^{35}S]methionine were from Amersham. PAI-2 mRNA (2–5 μg/ml) was translated in the presence of 110 mM potassium acetate and 3.5 mM magnesium acetate, including the contribution of the microsomes (30% final concentration) when present. TPCK-trypsin was from Sigma.

Isolation of a murine PAI-2 cDNA

Resident macrophages (1.5 × 10^8 cells) were isolated from the peritoneal cavity of 3-month-old AKR mice and selected by adherence overnight (Wohlwend *et al.*, 1987b). The cells were stimulated for 3 h with 1 μg/ml endotoxin, which increases PAI-2 production (Chapman and Stone, 1985) and mRNA content (A.Wohlwend, unpublished). Total RNA was isolated and the poly(A)-containing fraction purified by oligo(dT)–cellulose chromatography (Belin *et al.*, 1984; Collart *et al.*, 1987). Double-stranded cDNA was synthesized using an Amersham cDNA synthesis kit, and large cDNAs were isolated by centrifugation (4.5 h at 59 000 r.p.m., SW60 rotor) through a 5–20% sucrose gradient. The cDNAs (4 ng) were tailed with dGTP, annealed to pUC19 DNA, previously tailed with dCTP at the *Pst*I site, and transformed into high efficiency competent DH5 cells (BRL). The library (2 × 10^4 clones) was screened by hybridization to the 800 bp 5′-proximal human PAI-2 cDNA fragment isolated from pDB4707. Three murine PAI-2 cDNA inserts were isolated, one of which extends into the 5′ untranslated region; restriction fragments of this insert were subcloned into pUC19. Nucleotide sequences were determined on plasmid DNA using the dideoxy chain termination method and Sequenase (USB).

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Note added in proof

The complete nucleotide sequence of the murine PAI-2 cDNA will appear in the EMBL GenBank and DDBJ nucleotide sequence Databases under the accession number X16490. The deduced protein sequence of murine PAI-2 will appear in the SWISS-PROT Database under the accession number P12388.